GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF RESEARCH ADMINISTRATION

RESEARCH PROJECT INITIATION

Date: 14 March 1973

Project Title: "Ecological Studies of a Subtropical Terrestrial Biome: Microbial Ecology"

Project No: G-32-605

Principal Investigator: Dr. Nancy W. Walls

Sponsor: Florida Power and Light Company

Agreement Period: From March 1, 1973 Until February 28, 1974

Type Agreement: Industrial Research Agreement

Amount: $90,000

Reports Required: Quarterly Progress (Oral)
Annual Report

Sponsor Contact Person (s):

Mr. Norris R. Kincaid
Director, Environmental Affairs
Florida Power and Light Company
P. O. Box 3100
Miami, Florida 33101

Assigned to: School of Biology

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GEORGIA INSTITUTE OF TECHNOLOGY

OFFICE OF RESEARCH ADMINISTRATION

RESEARCH PROJECT TERMINATION

Date: April 21, 1975

Project Title: Ecological Studies of a Subtropical Terrestrial Biome: Microbial Ecology

Project No: G-32-605

Principal Investigator: Dr. E. L. Fincher & Dr. N. W. Walls

Sponsor: Florida Power & Light Company; Miami, Florida 33101

Effective Termination Date: 2/28/75 (End of second year)

Clearance of Accounting Charges: by 2/28/75*

Grant/Contract Closeout Actions Remaining: XXX

(1) If applicable, submission of Invoice for 2nd year charges not previously billed.
(2) Annual Report No. 2.

*Third year funding budgeted under account numbers G-32-620 & G-32-315 effective 3/1/75.

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G-32-605

ANNUAL REPORT

March 1, 1973 - February 28, 1974

Project No. G32-605

"ECOLOGICAL STUDIES OF A SUBTROPICAL TERRESTRIAL BIOME: MICROBIAL ECOLOGY"

Submitted to: Florida Power & Light Company
P. O. Box 3100
Miami, Florida 33101

Report Prepared by: Edward L. Fincher, Ph.D.

School of Biology
Georgia Institute of Technology
Atlanta, Georgia 30332

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ACKNOWLEDGMENTS

Acknowledgment is made of the professional contributions of Mrs. Dorothy E. DeFoor, Assistant Research Scientist, Dr. Royce N. Bramlett, Research Scientist, and Mr. Steven E. Ehlers, Assistant Research Scientist, to the planning and research activities of this study during the period covered by this report.

CONTENTS

I. INTRODUCTION

1.1 Project Objectives - The project objectives pursued within the period covered by this report were in accordance with the phasing of the project as outlined in the original proposal of the projected five-year study of the microbial (bacterial) flora at selected sites within the experimental study area of the Florida Power and Light Company property at Turkey Point. Objectives during this current period included principally the following:

(a) To conduct and complete a literature survey of information available in publications on the interactions and identity of bacterial species found in soils, fresh-water habitats, and salt-water estuaries,

(b) To establish research laboratories, acquire equipment and supplies pertinent to the implementation of the research plan,

(c) To locate and engage professional and/or technical personnel as required, and

(d) To begin isolation of bacteria from soil, fresh water, and salt water locations at the Turkey Point study site.

1.2 Project Background and Organization - This research is designed to contribute to the broad and comprehensive study of the environment at the Turkey Point facility by providing a detailed study of aerobic and anaerobic bacteria present in the soil and water, of changes in the bacterial flora with seasonal variation, and to determine the ecological role of bacteria through contributions made by these organisms to the total ecosystem. Since microbial forms, which include bacteria, are at the base of the primary

food chain in the cycling of carbon, nitrogen, and minerals, it is considered that the detailed knowledge of the bacterial ecosystems present would make an important contribution to understanding the dynamics of the total biological system operating in the particular environment under study.

Organization of the project and design of experimental work is directed first to the determination of the numbers and kinds of bacteria present in the soil and water. Quantitative determination of bacteria present in soil and water is difficult from the standpoint of selecting methods of isolation for unknown bacteria whose physiological and nutritional requirements are also unknown.

Isolation procedures are available for selective cultivation of certain types of bacteria in soil and water, but these methods are principally qualitative and restrictive in view of the probable numbers of different kinds of bacteria present and their unknown relationships to the assimilation and dissimilation of organic materials derived from higher life forms. These heterotrophic bacteria will be examined early in the study, but a parallel determination will be made of autotrophic bacteria as well. The determination and significance of total numbers of bacteria in the soil is viewed with some variance of opinion by workers in soil bacteriology, but it is felt that quantitation of bacteria present in soil, particularly, and in water is important to the objectives of this study in providing some measure of the adequacy of techniques used in isolation of the bacteria. Also, this will provide one form of an index that might make it possible to determine the effect of various imposed environmental stresses on the bacterial ecosystem.

1.3 Methodology - Implementation of the design of the study will be done through periodic visits to the field study-site for the purposes of obtaining samples of soil and water for bacteriological analysis in laboratories of the School of Biology at Georgia Tech. Logistical problems are associated with processing samples from collection sites remote from the processing laboratories with minimum delay to avoid alteration or loss of the bacteria present. This difficulty is compounded in the case of the anaerobes which must be protected from exposure to oxygen in transit. Determining optimal methods to solve these problems has been an important consideration in this first effort.

Procedures for separating the bacteria from soil samples, or from water, and their characterization by an extensive array of physiological and biochemical criteria will be done by published methods and, when required, by such methods as may be developed in the laboratories here. A large number of different bacterial isolates are expected to be generated by this search. Each isolate will be characterized by 150-200 individual character "bits"; the resulting extensive data accumulated will be analyzed by computer methods.

Core samples of soil will be characterized by their geological identities and subjected to chemical analysis, the results of which will be important data as possibly correlatable with the qualitative analysis of bacteria present in various strata or depths of the soil. Discussions have been held with staff members of the School of Geophysical Sciences, Georgia Tech, who will conduct these particular characterizations and analyses.

The important second phase of the study will be devoted to a major effort in determining the physiological and nutritional inter-

actions between components of the bacterial ecosystem for the purpose of detecting the nature and magnitude of dominant sub-population pressures and their ecological role in this presently non-impacted environment. This second phase will be undertaken after the bacterial types and their distribution in soil and water are determined and after some determinations are made of the relative qualitative and quantitative stability of the sub-populations which analysis indicates to be significant.

II. PROGRESS STATUS

2.1 Preliminary Bacteriological Analysis of Soil - Several questions had to be answered before an extensive and prolonged analytical procedure could be applied to determining the kinds and distribution of bacteria present in soil samples. Important questions related to (a) how many bacteria were present, (b) what were the numbers of bacteria at different depths, (c) what medium would recover the greatest number of bacteria and most diverse types, and (d) what culture conditions of pH, oxygen tension, and temperature should be selected. Data have been obtained that provide the basis for the more detailed methods of analysis.

2.1.1 Methods - Bacteriological

(i) Primary Isolation Media - Reports of culture media for initial recovery of a broad group of soil bacteria were compiled from published literature sources and studied to determine what common characteristics existed among the media. This was done to select the minimum number of different media required for optimal recovery of aerobic heterotrophic bacteria.

Three media were selected on this basis: (1) Trypticase Soy Agar - Yeast Extract (0.2% wt./volm.); (2) Soil Extract Agar - Yeast Extract (0.2% wt./volm.); (3) Yeast Extract (0.2% wt./volm.) Agar. Agar concentration in all media: 1.5% (wt./volm.).

The soil extract was prepared according to the schema shown in Figure 1. The filtrate alone had a pH of 5.8 (pHydrion paper).

The media used in the qualitative recovery of anaerobic bacteria from soil samples were (1) Cooked Meat Medium (Difco); (2) Sulfate Reducing Medium; (3) ZoBell's Medium 2216E with soil extract in the place of sea water plus 1% agar; (4) ZoBell's Medium 2216E with deionized water plus 1% agar.

(ii) Incubation Temperatures - Two temperatures were selected for cultivation of the aerobic heterotrophic bacteria: 18 C and 30 C. For cultivation of anaerobes the temperature was 18 C.

(iii) Separation and Processing Methods - Separate sections (1 cm) of the cylindrical (1-7/8" diameter) soil core sample were disbursed and thoroughly mixed in 10 ml Trypticase Soy Broth, 1% (BBL), and then diluted 10^{-2}, 10^{-3}, and 10^{-4}. From these, 0.5 ml, 0.1 ml, and 0.5 ml respectively were streaked on the surface of the various primary siolation media. A schematic display of this procedure is shown in Figure 2 for the aerobic bacteria.

To cultivate anaerobic bacteria at each core level tested, approximately 1/10 of the 1 cm slice of the soil core was aseptically transferred to each of the media described above

FIGURE 1.

PREPARATION OF SOIL EXTRACT

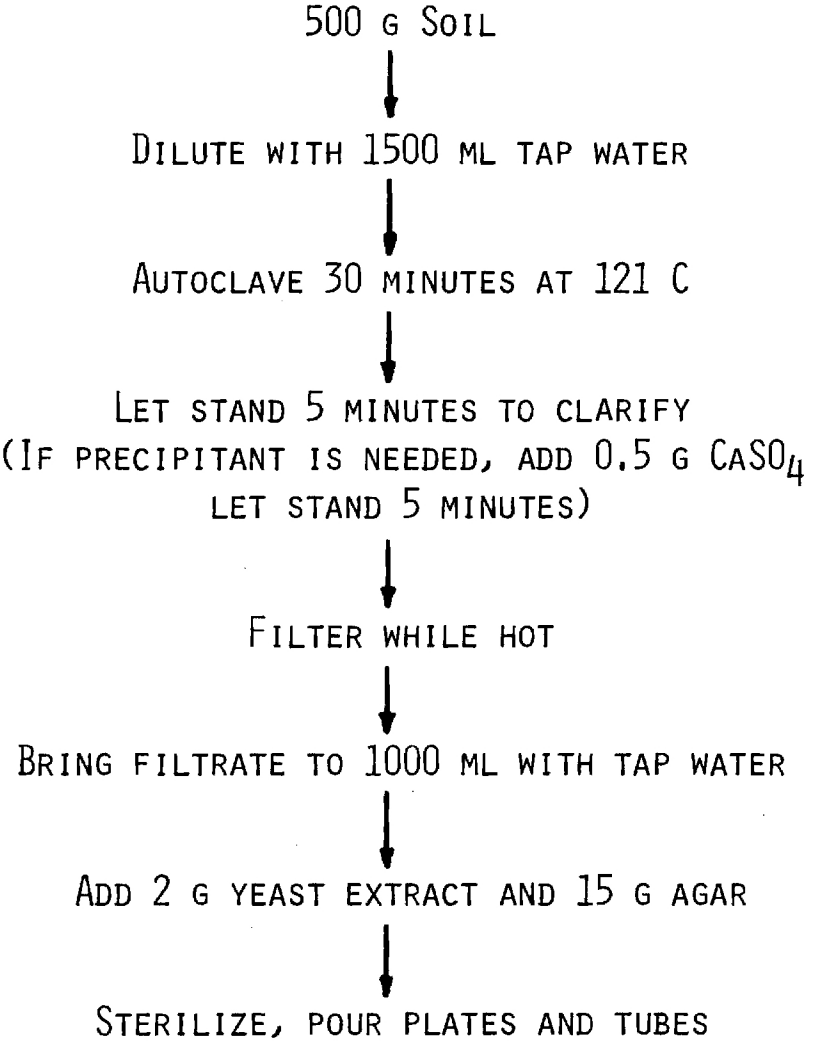
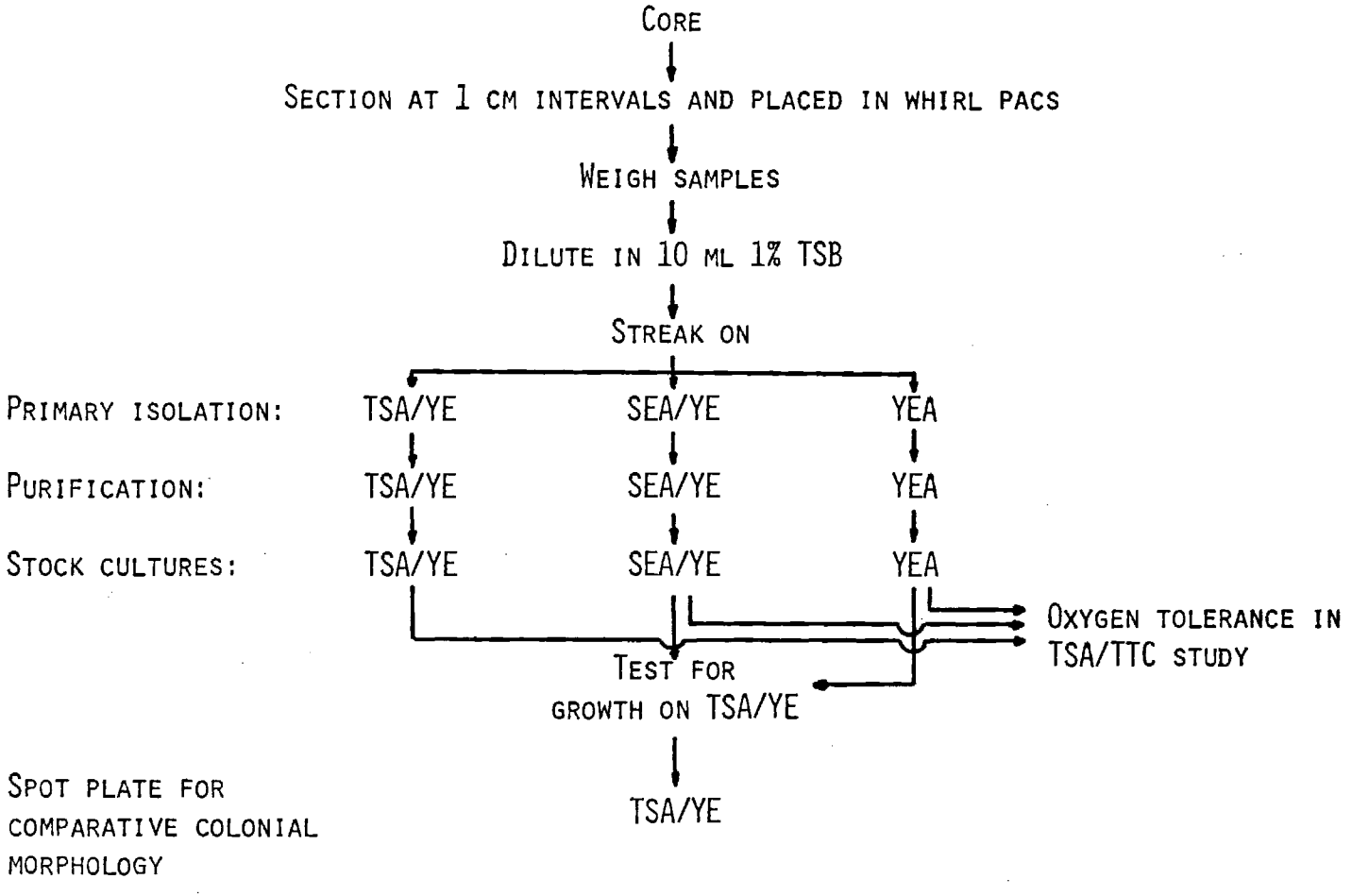


FIGURE 2.

FLOW-SCHEMA FOR ISOLATION OF HETEROTROPHIC SOIL BACTERIA



(duplicate tubes for cooked meat medium). One set of cultures in cooked meat was heat shocked prior to incubation to facilitate recovery of spore-formers. All cultures were incubated at 18 C in GasPak jars in an atmosphere of hydrogen and carbon dioxide.

2.1.2 Methods - Soil Samples

(i) Soil samples were taken on January 16, 1974 at a point approximately 100 meters from the northerly end of the sampling grid line at the study-site. Samples were obtained with a Wildco Hand Corer* which can recover a cylindrical soil core 1-7/8" x 20". These cores were taken between 8:30 AM and 10:30 AM, transported in an upright position in a special carrier constructed for the purpose, and processed at Georgia Tech within 14 hours after the samples were taken.

The soil core sample was extruded intact from the plastic core sampler tube and sectioned at 1 cm intervals. Each section was then divided approximately equally, and one-half section was placed in a sterile Whirl-Pak plastic bag to which was added 10 ml of 1% TSB (Figure 2) for bacteriological analysis; from a second soil core a one-half section was placed in a Whirl-Pak bag for determination of moisture content. The average wet weight of the one-half sections was 10.7 $\pm$ 0.9 gms (range: 9.0 - 13.3 gms) each.

2.1.3 Results

(i) Soil samples - Extruded cores of soil samples examined in the laboratory showed four distinctive zones of interest:

*Model #2422, Wildco Instruments, 301 Cass Street, Saginaw, Michigan, 48602.

Zone 1 (1 cm Sec.) - Surface of detritus of plant origin, and a greenish film-like layer suggestive of dried algae.

Zone 2 (2-12 cm Sec.) - A grey, marl-like section that appeared to be composed of finely divided clay-type material, very homogeneous, indicating a sedimented deposit.

Zone 3 (13 cm Sec.) - A section showing undecomposed detritus of unknown age.

Zone 4 (14-25 cm) - Granular earth in composition in the upper portion, graduating into a dark, gluey or viscous terminal portion.

A one-half section (10 gm) of each successive 1 cm soil slice, to a total depth of 25 cms, was mixed individually with 10 ml distilled water and the pH determined to be 8.0 $\pm$ 0.2 with a pH meter.

An approximate determination of the moisture content was made with the result that the dry weight:wet weight = 0.45. This measurement was made on a core after its storage in a vertical position for 5 days and therefore did not duplicate conditions in the field.

(ii) Bacteriological Analysis

(a) Primary Isolation Media - Results reported in Table 1 indicate that recovery performance of Trypticase Soy Agar/Yeast Extract (TSA/YE) is equivalent or superior to Soil Extract Agar/Yeast Extract (SEA/YE). These data do not indicate if the bacteria recovered are identical; however, transfer of isolates from SEA/YE to TSA/YE showed that this latter medium sustains growth satisfactorily, only one isolate failing to grow on TSA/YE.

TABLE 1.

AVERAGE NUMBER OF HETEROTROPHIC BACTERIA
PER GRAM (DRY WEIGHT) OF SOIL

	SOIL DEPTH$_{(CM)}$									
MEDIUM	1	2	3	5	7	9	13	17	21	25
TSA/YE	4.0**	2.2	2.2	3.3	0.33	NC*	-	1.7	1.2	5.4
SEA/YE	NC	NC	1.1	1.7	0.41	NC	-	3.2	3.2	2.7
YEA	0.36	0.74	0.26	0.23	0.06	0.23	-	0.22	-.13	0.35

INCUBATION AT 18 C. FOR 5 DAYS.

*NC - NOT COUNTABLE - PLATES OVERGROWN.

**NUMBER OF BACTERIA X 10^6 ORGANISMS/G.

TSA - TRYPTICASE SOY AGAR

YE - YEAST EXTRACT

SEA - SOIL EXTRACT AGAR

It was quite clear that the media selected for cultivation of the anaerobes supported luxuriant growth of a number of organisms. The separation of anaerobes from facultative organisms is a tedious undertaking which is still in progress, but some results are evident:

(1) Two strict anaerobes have been isolated from cooked meat cultures, one at 23 cms and the other at 25 cms. The former is a short, slender bacillus and the other is a spore-former. Additional attempts at isolation of anaerobes from this medium are continuing.

(2) Blackening in the sulfate reducing medium, taken as presumptive evidence of sulfate reduction, occurred at 2, 6, 7, 12, 15, 18 and 20 cms. Isolation and identification of the organisms involved are also in progress.

(3) The cultures in ZoBell's media are stored as deep stabs, awaiting further study.

(b) Distribution of Number of Bacteria with Soil Depth - In general, the number of bacteria found decreases with increasing depth. At or below 13 cm a slight rise in numbers is observed which, however, does not approach the counts obtained in upper sections of the core. Unfortunately, the presumed critical 13 cm section was lost due to unsatisfactory processing. The total number of bacteria isolated in 1-25 cm of soil ranges from 0.33-4.0 x 10^6 per gm dry weight of soil (Table 1).

(c) Oxygen Requirement of Bacteria and Soil Depth - Inoculation of deep tubes (10 cm) of TSA containing 0.1% triphenyl tetra-

zolium chloride (TTC), a redox indicator, indicates that strict aerobes occur at a relatively low frequency but are found at nearly all depths, including a depth of 25 cm; the microaerophiles tend to dominate in ratio at 3-5 cm depths, occur in significant numbers at depths of 2-17 cm, and with low frequency at 21 cm.

The aerobe-facultative anaerobes, classified on the basis of first growth appearing at the top (aerobic) of the redox growth tube at 24 hours and followed by deep (facultative anaerobic) growth at 48 hours, are dominant at the 1 cm soil depth and decline in number as a function of depth. This decline reaches a plateau at approximately 5-9 cm, below which they occur at relatively constant frequency to a depth of 25 cm in the soil.

The facultative anaerobes, classified on the basis of first growth appearing at the lower end (anaerobic) of the redox growth tube at 24 hours followed by progressive growth in the upper (aerobic) portion of the tube and/or those isolates that grew initially throughout the tube. This group of bacteria shows an inverse incidence with depth as compared to the aerobic-facultative anaerobes.

A graph of these results is shown in Figure 3.

(d) Gram Reaction of Isolates - Figure 4 shows that the dominant bacteria isolated aerobically were gram-negative bacilli which occur at all depths. At 1 cm depth all of the 8 different bacteria isolated were gram-negative bacilli; at depths of 2-25 cm, they occur with a frequency of approximately 75%. The gram-positive bacilli are found within a frequency of 10-20% in the soil depth range of 2-25 cm.

FIGURE 3.

OXYGEN REQUIREMENT OF BACTERIAL ISOLATES AS A FUNCTION OF SOIL DEPTH

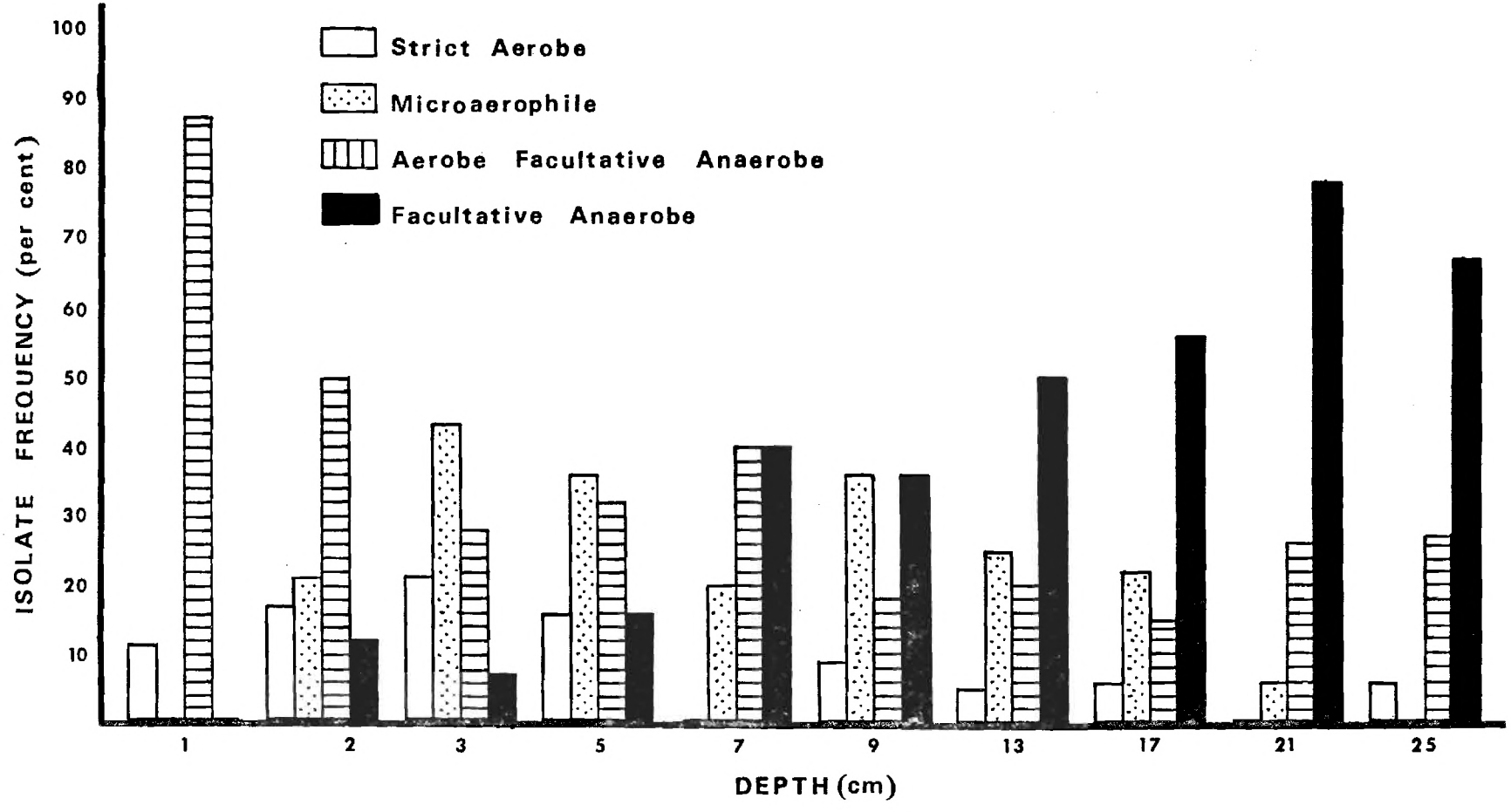
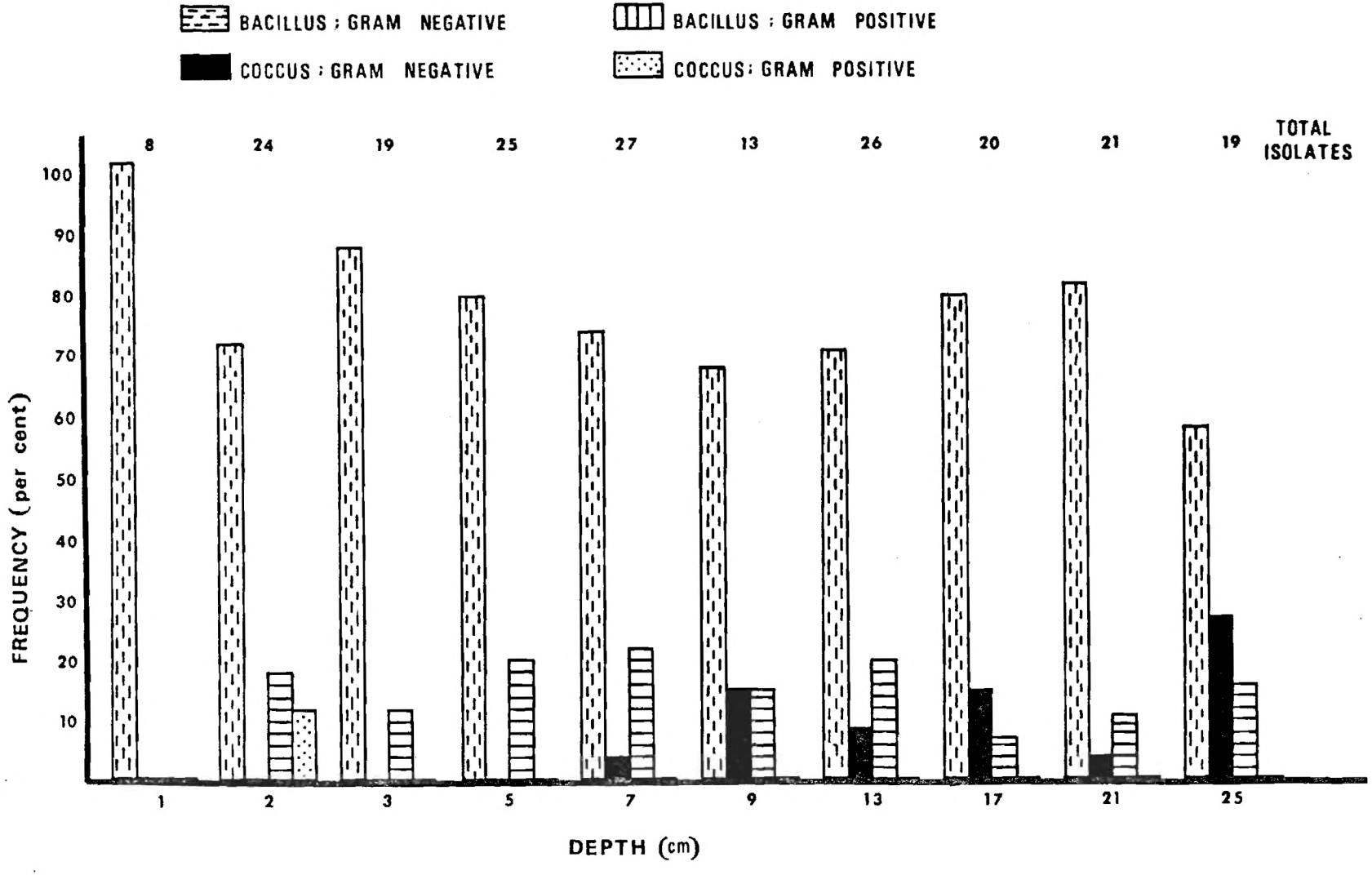


FIGURE 4.

GRAM REACTION, BACILLUS/COCCUS DISTRIBUTION AS A FUNCTION OF SOIL DEPTH



Gram-negative cocci are first noted at the 7 cm depth and occur at all subsequent depths, approaching 30% of the 19 bacteria isolated at 25 cm.

NOTE: Results reported in Figures 3 and 4 were from studies on pure cultures of bacteria, cultures determined by a subjective evaluation of probable different types of bacteria based on differing colonial morphology on TSA/YE and SEA/YE.

(e) Temperature of Incubation - Observable growth occurred at 30 C within 48 hours but with concurrent problems of actively motile bacteria over-growing adjacent colonies on the agar surface. At 18 C, 5 days incubation, the qualitative assessment of growth indicated the same organisms seen at 30 C but without the swarming and over-growth by motile organisms. This temperature of 18 C was also satisfactory for the anaerobes.

2.1.4 Conclusions

(1) Aerobic and facultative anaerobic bacteria occur to depths of at least 25 cm, the total number being in the range of 0.3-4.0 x 10^6 bacteria/gm of soil (dry wt.); dominant forms are gram-negative bacilli, followed by gram-positive bacilli and gram-negative cocci--in that order.

(2) The TSA/YE primary isolation medium appears adequate for isolation of heterotrophic bacteria demonstrable at 18 C or 30 C incubation temperature.

(3) The vertical depth of soil shows 4 zones of grossly determinable strata which may support different types and numbers

of bacteria, the latter possibility being suggested by differences in numbers at varying depths (Table 1).

(4) Anaerobic sulfate-reducing bacteria seem to occur throughout the depth of the soil column, from 2 cms through 20 cms. Isolation of the anaerobes is proving rather difficult under the physical limitations imposed by the use of GasPak jars, but should proceed rapidly with the acquisition of the anaerobic glove box.

2.2 Numerical Taxonomy

An essential requirement of the project is handling the mass of descriptive data which will be used to characterize the different bacterial isolates and to determine their frequency of occurrence. To meet this requirement, the techniques of numerical taxonomy are being utilized which can only be implemented through the use of high-speed electronic computers. The UNIVAC-1108 is being used in this study.

At the present time computer programs have been developed to calculate the similarity index (S_{sm}) of different bacteria (OTU's) and arrange the OTU's in sub-group clusters within a matrix. Part of this procedure is yet dependent on manual methods which are laborious and time-consuming. A partially developed solution to computer clustering of OTU's has been realized in a program that will produce a reasonably satisfactory clustering of OTU's forming clusters.

What is ultimately desired for application in this study is a computer program that will construct sub-groups by the average linkage method, the admission of individual OTU's being determined by pre-set limits, the computation of dispersion values of sub-groups, and the determination of distance values between sub-groups.

A program is available for computer print-out of inter-group relationships in an easily read dendritic form.

2.3 Project Personnel

In addition to Edward L. Fincher, Ph.D. (Bacteriologist) and Nancy W. Walls, Ph.D. (Bacteriologist), co-principal investigators of this project, the following personnel have been added to the project staff on the indicated dates:

(1) Steven E. Ehlers, M.S. (Bacteriologist), Assistant Research Scientist, May 1, 1973.

(2) Dorothy E. DeFoor, B.S. (Bacteriologist), Assistant Research Scientist, July 1, 1973.

(3) Royce N. Bramlett, Ph.D. (Biochemist), Research Scientist, December 1, 1973.

2.4 Research Laboratories

Research laboratory space requested in addition to the one (1) medium size and one (1) small size laboratories available at the inception of this study has not become available at the time of this report. Additional laboratory space has been forecast for occupancy on May 1, 1974 and will significantly alleviate the limitations and congestion of facilities now utilized in this research. Until these additional facilities are in operation, the full effectiveness and scope of planned activities cannot be realized. In the meantime, however, progress in approaching the objectives outlined will not be seriously impaired.

Essential equipment and supplies for this additional research facility have been acquired and are available for present use. Specifications for an anaerobic glove box roughly 4' x 8' have been approved and an order has been submitted for this structure which will contain

storage shelves for prepared media, a small incubator, and other equipment for transfer and maintenance of anaerobes. Upon delivery, activation of the glove box must await space now used while a larger laboratory is being altered and fitted for use.

2.5 Literature Review

A comprehensive search of published literature for research results relevant to objectives of this study was made during the first six months of the first year of this project. An edited list of these publications which are considered most pertinent appears in the Appendix.

A continuing review of the literature will be a part of future efforts in this study, periodically examining most recent published material for interest and utility in this research.

III. PROPOSED STUDIES - SECOND YEAR

Preliminary research design undertaken during the first year of this study will be continued with major direction taken in the following investigations:

1. Quantitative and qualitative analysis of aerobic bacteria present at various depths in soil core sampels taken at 100-150 meter intervals along the established sampling grid at the Turkey Point site. Isolation procedures will be directed toward recovery of the maximum number of bacteria cultivable under heterotrophic conditions. The schedule of analyses includes the initiation of special isolation procedures and enrichment culture methods, in approximately 5-7 months, to qualitatively determine the aerobic bacterial flora that may be recovered by heterotrophic culture methods.

2. Parallel to the bacteriological analysis outlined in Part 1 (above) is the continued development of computer programs to be used in determining the numerical taxonomic relationships between the bacteria

recovered. Existing programs employed in determining coefficients of similarity between individual bacterial isolates are to be extended to machine computational methods in constructing matrices representing bacteria grouped by average linkage of similarity, accompanied by measures of statistical variations or dispersion in related groups. Further program development will be sought to display this information as machine print-out in the form of dendrograms. This approach should enable identification of principal bacterial groups and their relative geometrical relationships, presenting a major progression in determining the bacterial ecosystem present in soil.

3. Instrumental and procedural capability will be developed within the March-April, 1974 period for the assay of adenosine triphosphate (ATP) in soil core sections. Data on the ATP content of different strata of soil between the ground surface and 30 centimeters, supplemented with appropriate viable cell data, are considered potentially useful as an index of total biomass which should provide a means of evaluating the effects of environmental, nutritional, or toxic variables on the total microbial life flux in the system.

Soil samples will be assayed for total ATP content which should be indicative of the total biomass. It may be possible that this data, in conjunction with enumeration of viable bacteria and direct microscopic bacterial cell count, can be used to estimate only the bacterial biomass. The total cellular organic carbon computed from total biomass data will be compared to the total organic carbon to yield a ratio of "living" organic content to total organic material.

Higher ATP concentrations are expected to be found where aerobic species predominate in activity since the efficiency of obtaining

energy in the form of ATP from substrates is approximately 10-fold greater in aerobic than in anaerobic processes. If the ATP concentration can be standardized and correlated with depth of the soil, which should also be proportional to the ratio of aerobic to anaerobic species, then large variations from these standard values could indicate changing metabolic patterns. From these data and estimations of total organic carbon, it should be possible to detect changes in biomass and metabolic activity caused by seasonal or other environmental change.

4. Qualitative determinations will be continued to assay the anaerobic bacteria present in soil cores. The inherent technical difficulties in isolating and culturing these bacteria restrict quantitative determination of the anaerobic bacteria at the present stage of this study. In the next 4-5 months, laboratory analysis will be limited to attempts to identify the organisms recovered. As experience and facility in the use of the projected anaerobic work-chamber is acquired, studies of methods will be commenced to determine the quantitative ratio of anaerobic to aerobic bacteria present in the soil core.

5. Preliminary experiments will be started in the latter part of the currently proposed support periods on measurements of rates of respiration of selected bacterial isolates representing dominant taxonomic groups. These experiments will also include initial studies on mixed cultures to determine in a simple system some of the factors operating in the bacterial ecology of the soil. These experiments will depend on completion of the numerical taxonomic grouping of the bacteria identified in Parts 1 and 2 above.

IV. APPENDIX

4.1 Results of Literature Survey

Bacteriological Methods - Aerobes

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